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In vivo distribution of arsenic after i.p. injection of arsonoliposomes in balb-c mice

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Abstract

We recently showed that arsonoliposomes (novel arsenic containg liposomes) demonstrate differential toxicity towards various types of cancer and normal cells, in cell culture studies, as well as anti-parasitic activity. In this study, the in-vivo distribution of the active moiety of these vesicles, As, is evaluated.

Sonicated arsonoliposomes were prepared using the arsonolipid with palmitic acid acyl chain (C16) mixed with egg-phosphatidyl choline (PC) and cholesterol (Chol) [C16/PC/Chol at 8:12:10 mol/mol/mol]. A dose of arsonoliposomes, corresponding to 5 mg arsenate/kg was administered by intraperitoneal injection in balb-c mice. At various time points post-injection the mice were sacrificed and the distribution of As in the organs was measured, by atomic absorption spectroscopy.

Results demonstrate that a high portion of the dose administered is rapidly excreted; since 1-h post-injection only about 30% of the dose administered was detected cumulatively in the animal tissues. After this the elimination of arsenic was a slow process with a total body elimination rate constant of $0.023\,h^{-1}$, corresponding to a half-life of 30 h. Tissues with the highest arsenic concentration during the study period are: spleen–kidneys–stomach, followed by lung, liver, intestines–heart, carcass + skin and finally blood. No acute toxicity, or effect on the body or organ weight of the mice was observed.

Keywords: Liposome; Arsenate; Arsenic; Distribution; Tissue; Mice; In vivo; Toxicity

1. Introduction

Recent clinical reports (Zhang et al., 1996; Soignet et al., 1998; Bode and Dong, 2002), indicate that arsenic trioxide (ATO) is extremely effective for inducing complete remission in patients with acute promyelocytic

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leukemia (APL), a rare, life-threatening form of cancer of the blood. As a result of these studies, a formulation of ATO (Trisenox®) has been approved for marketing in 2000 by the U.S. Food and Drug Administration (to treat patients with APL), and was recently (March 2002) granted marketing authorization from the European Commission. Nevertheless, a significant drawback for the broader use of arsenic(III) based compounds in other than salvage therapy applications, is their high toxicity (Huff et al., 1999; Westervelt et al., 1997). ATO has been demonstrated to reduce viability of endothelial cells at relevantly low concentrations in a time and dose dependent manner (Roboz et al., 2000). Data from several studies prove that arsenic trioxide is active (in APL) at doses ranging from 0.06 to 0.20 mg/kg. Attempts to increase the dose beyond this range (Westervelt et al., 1997) resulted in severe toxic reactions, including flaccid paralysis and renal failure.

A general technological approach for reducing drug toxicity that has worked well in several instances (especially with cytotoxic drugs) is the inclusion of the highly toxic substance in stable liposomal formulations (Gregoriadis, 1993; Lasic and Papahadjopoulos, 1998). This may result in reduced toxicity and simultaneous enhanced activity of the drug substance, if of course the kinetics and distribution of the drug is altered, which is the case when the drug is sufficiently retained by the vesicular carrier. In this respect, we recently investigated the possibility of preparing liposomal formulations of ATO (Kallinteri et al., 2004). However, although high concentrations of ATO were initially encapsulated in large multivesicular liposomes, all liposomal formulations evaluated demonstrated rapid leakage of ATO, in vitro. Even vesicles composed of saturated lipids mixed with cholesterol which, are known to form rigid and stable liposomes, were not able to retain high amounts of ATO, in vitro, leaving small chances for any application of such formulations.

An alternative method to deliver arsenic compounds by vesicles, would be the preparation of liposomes incorporating arsenic-containing lipids, as arsonolipids. Indeed, we recently prepared and characterized in our laboratory arsonoliposomes or arsonolipid-containing liposomes (Fatouros et al., 2001). Arsonoliposomes have been characterized in respect to their size and surface charge, their ability to encapsulate hydrophilic molecules and their in vitro stability in buffer as well as in the presence of serum proteins.

Promising results were obtained with some of the arsonoliposomes prepared, for which a differential toxicity towards cancer and normal cells was demonstrated (Gortzi et al., 2002, 2003) as well as in vitro antiparasitic activity (Antimisiaris et al., 2003). Thereby, it is essential to begin studying their in vivo behavior. This was attempted previously, however, the analytical technique used (XRF) was not sensitive enough for determination of As in all tissues. In the present study, after setting up a sensitive atomic absorption technique for the determination of As in biological tissues using a stabilized temperature platform furnace, we studied the in vivo distribution of arsenic, the active moiety of these vesicles, after intraperitoneal administration in balb-c mice. For this, we used the palmitic acyl chain arsonolipid, which was selected after taking into account the results of in vitro stability and cell culture studies, and liposomes composed of C16-arsonolipid, egg-PC and cholesterol, at a C16/PC/Chol 8:12:10 mol/mol/mol ratio were prepared, characterized and administered by intraperitoneal injection in female balb-c mice. The distribution of arsenic in various organs at various time points post-injection was determined.

2. Materials and methods

2.1. Materials

Egg L-α-phosphatidylcholine [PC] (grade 1) was obtained from Lipid Products, Nutfield, UK. The 99% purity of the lipid was verified by thin layer chromatography on silicic acid-coated plates (Merck, Darmstadt, Germany) using chloroform/methanol/water (65:25:4 v/v/v) as the solvent system and iodine staining (New, 1990). The lipid used gave single spots. Cholesterol [Chol] (pure), all buffer salts and all other reagents used (analytical grade) were obtained from Sigma-Aldrich (OM), Athens, Greece. The water was deionized and then distilled (DDW).

The *rac*-arsonolipid [Ars] (2,3-diacyloxypropylarsonic acid) with a palmitic side chain ($R = C_{15}H_{31}$) was synthesized and characterized, as described in detail before (Tsivgoulis et al., 1991a, 1991b; Serves et al., 1992, 1993).

2.2. Preparation of arsonoliposomes

Liposomes containing mixtures of Ars with phosphatidylcholine [PC] (40% Ars or Ars/PC/Chol

8:12:10 mol/mol/mol) were prepared as reported previously (Fatouros et al., 2001) by sonication after initial dispersion of the lipids in phosphate buffer saline (PBS) which was achieved by magnetically stirring at 70 °C (above the Ars transition temperature) for 4 h. Two 5-min sonication runs were performed, separated by a 10-min interval, using a probe type sonicator (Sonics and Materials, UK). After sonication the liposomes were centrifuged for 10 min at 10,000 rpm in order to remove titanium pieces and liposome aggregates which could be present in the samples, and left in peace for 1 h for annealing of any structural defects. The lipid content of the samples was determined using a colorimetric technique, which is widely applied for phospholipids, the Stewart assay (Stewart, 1980), in which the ability of phospholipids to form a complex with ammonium ferrothiocyanate in organic solution is utilized. In brief, liposome samples (20 μL) are vortexed with 2 mL of a solution of ammonium ferrothiocyanate (0.1 M) and 2 mL of chloroform. The OD 485 nm of the chloroform phase is measured and the lipid concentration of samples is calculated by comparison with an appropriate standard curve.

The arsenic and – after calculations – arsonolipid content of the vesicles was determined by the atomic absorption technique presented below (Section 2.5).

2.3. Liposome characterization

The liposomes prepared were characterized by measuring their size distribution by PCS (photon correlation spectroscopy) with a Malvern Zetasizer 5000 (Malvern, UK), as described before (Fatouros et al., 2001). In brief, liposome dispersions were diluted with filtered PBS pH 7.40 and sized immediately.

The arsonoliposome electrophoretic mobility was also measured at 25 °C (Zetasizer 5000 Malvern Instruments, UK), after diluting the vesicle dispersion with filtered buffer. Zeta potentials of the dispersions were calculated (by application of the Helmholtz–Smolowkovski equation).

2.4. In vivo distribution study

Twenty-six female balb-c mice with a mean weight of 19.6 ± 1.9 g from the University Hospital animal facility were used. The animals were housed in an approved animal facility and maintained according to the National Institutes of Health "Guideline on the Care and Use of Laboratory Animals". The animals were provided with Rodent Chow and water ad libitum. Two hundred microliters of a palmitic acid arsonoliposome dispersion (Ars/PC/Chol 8:12:10 mol/mol/mol), which corresponds to 1.5 mg of total lipid (arsonolipid and PC) or 100 μg of As(V) (approximately 5 mg/kg As(V)), were injected i.p. to each animal. At specified time points post-injection (1, 3, 5, 7, 12 and 24 h), animals were sacrificed in groups of four, by decapitation. Two animals were sacrificed without treatment (their tissues were used as blank controls, and for preparation of control spiked samples). Blood samples were immediately taken from each animal by cardiac puncture. After this, the animal tissues [liver, kidneys, spleen, lungs, heart, intestines (small and large together), stomach and carcass + skin] were removed from the animals and weighted.

The As content of all tissues was measured by the technique described in detail below.

2.5. Quantification of arsenic in tissues and arsonoliposomes

2.5.1. Sample preparation

The arsonolipid content of arsonoliposomes as well as the tissue concentrations of arsenic were determined using atomic absorption spectrophotometry after digestion with concentrate nitric acid, as previously reported (Desaulniers et al., 1985). In brief, each tissue from each mouse after being weighted (or 0.5 g from large tissues) was dried and placed in a 250 mL Erlenmeyer conical flask with 10–50 mL of fuming HNO_3 (depending on the weight of each tissue). For determination of arsenic concentration of arsonoliposomes, 20 μL of the liposome dispersion were digested in 2 mL of nitric acid. The flasks were heated on hot plates placed under a hood, by slowly increasing the temperature to 90–100 °C. The solution was allowed to evaporate to dryness (but not charred), and the residue was taken up with 3 mL HNO_3 and 3 mL cold (4 °C) 30% H_2O_2. A reaction was initiated by slowly heating the mixture and the rate of decomposition of H_2O_2 was controlled by frequently removing the flask from the hot plate. The solution was then brought to a brief boil, cooled, and diluted to 50.0 mL with DDW. Blanks were taken through all steps.

2.5.2. Procedure

The total arsenic in these samples was determined by graphite furnace atomic absorption spectroscopy technique (GFAAS). A computer-controlled atomic absorption spectrometer (AAnalyst 300, Perkin-Elmer) equipped with a graphite furnace (HGA-800, Perkin-Elmer) was used in this study. The absorption was measured at a wavelength of 193.7 nm and a 0.70-nm slit bandwidth. Deuterium lamp continuous background correction was used throughout the study to eliminate spectral interferences. Pyrolytic graphite-coated tubes (Perkin-Elmer) were used and the atomization process was done at the tube wall. Argon at a 250 mL/min flow rate was used as a purge gas and its flow was interrupted during atomization.

Matrix modifier solution of nickel nitrate (5% m/V) was prepared by dissolving an appropriate amount of the corresponding high purity salt (+99.999%, Aldrich) in water. The addition of matrix modifier converts the As to a less volatile compound and thus the char temperature may be increased to 1400 °C. An aqueous arsenic standard solution of 1000 mg/L (Merck) was used for the preparation of aqueous calibration standards of lower concentrations (20–300 ppb). These standards were prepared daily, acidified with nitric acid and stored in polyethylene containers. The final nitric acid concentration was 0.2% (m/V). The furnace conditions during the analysis are summarized in Table 1. The linear range was found to be 0–400 ppb. A typical calibration curve obtained by this technique is presented in Fig. 1.

The reagent blanks (0.2% nitric acid) and calibration standards were measured in duplicate, while samples in quadruplicate. A 10-μL aliquot of sample/standard was transferred on to the wall of the pyrolytic graphite-coated tube, followed by 10 μL of matrix modifier solution. The arsenic in the samples/standards was atomized by raising the temperature from ambient to atomization-temperature, according to the temperature program presented in Table 1.

Table 1
Heating cycle conditions (ramp time, hold time and temperature), during analysis of the tissue samples

Heating cycle	Ramp time (s)	Hold time (s)	Temperature (°C)
Dry I	5	30	90
Dry II	5	60	150
Ash	3	20	1400
Atomize	0	5	2300
Clean	0	5	2600

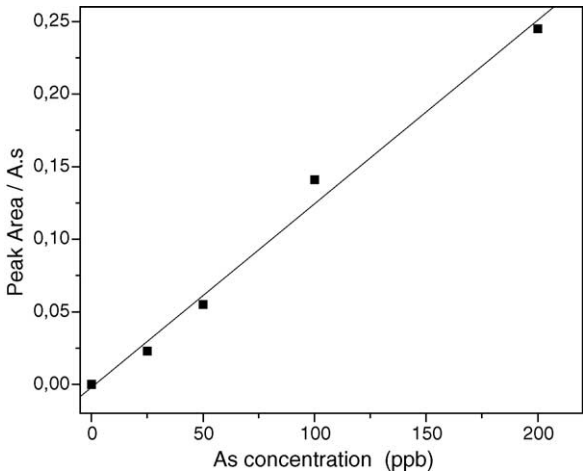

Fig. 1. Typical calibration curve of arsenic measured by the atomic absorbtion technique presented in detail in Section 2.5.

The reliability of the measurement was assessed by measuring blank tissues (from the animals that were not injected with arsonoliposomes) and by spiking control tissue samples with known amounts of arsonoliposomes or inorganic arsenic. Recoveries in all cases ranged from 88 to 109% with coefficients of variations between 4 and 12%.

3. Results

The arsonoliposomes used in the in vivo study were characterized by measuring their size distribution and surface charge. The results of these measurements: 91.5 ± 4.2 nm (mean diameter) and $(-)45.3 \pm 2.6$ mV (ζ-potential), are in absolute agreement with previous findings (Fatouros et al., 2001).

The tissue-distribution data of arsenic in the mice at various time points after intraperitoneal administration of arsonoliposomes are shown in Table 2. At the first time point, 1-h post-injection, the distribution of arsenic was greater in the carcass and skin samples, followed by, in descending order: intestine, liver, stomach, kidney, spleen, lung, heart. After this, the relative distribution of arsenic between the organs fluctuates, while for all the period studied the organs in which less arsenic was distributed (% dose/tissue) are the lung and

Table 2
Distribution of As in tissues and carcass + skin of balb-c mice at various time points following i.p. administration of arsonoliposomes (containing 5 mg/kg arsenate)

Time (h)	Liver	Kidneys	Lungs	Spleen	Heart	Stomach	Intestines	Carcass and skin
1	2.54 (0.41)	1.55 (0.10)	0.247 (0.018)	0.72 (0.12)	0.108 (0.021)	2.39 (0.68)	3.58 (0.84)	13.71 (2.36)
3	2.10 (0.50)	2.45 (0.38)	0.336 (0.081)	0.523 (0.075)	0.084 (0.022)	1.41 (0.23)	0.550 (0.081)	10.92 (1.60)
5	2.364 (0.072)	1.49 (0.26)	0.313 (0.063)	0.693 (0.082)	0.148 (0.027)	1.49 (0.30)	1.038 (0.062)	10.73 (2.03)
7	1.74 (0.31)	1.190 (0.070)	0.525 (0.048)	0.72 (0.12)	0.227 (0.039)	0.99 (0.24)	5.17 (0.62)	11.61 (2.47)
12	0.913 (0.034)	0.742 (0.094)	0.313 (0.044)	0.81 (0.13)	0.1152 (0.0074)	1.27 (0.25)	5.33 (1.09)	12.40 (2.07)
24	0.259 (0.039)	0.638 (0.076)	0.175 (0.039)	0.366 (0.057)	0.122 (0.017)	0.604 (0.078)	3.87 (0.14)	BDL

BDL: below detection limit. Results are expressed as mean% of dose/tissue (±S.D.). Each point is the mean of four values.

heart. Brain samples from all animals were analyzed and no arsenic was detected, in any case.

When the mean value of the total amount of arsenic present in the animals (in all tissues) at the various time points tested are plotted against time (Fig. 2), it is evident that the kinetics of arsenic clearance from the mice is bi-exponential. This is probably due to the mode of injection used here, intraperitoneal injection, which allows fast distribution to the organs involved in the clearance of xenobiotics, i.e., liver, kidney and bile. Thereby already at 1-h post-injection only about 30% of the total dose administered is cumulatively present in the animal tissues. Nevertheless, after this rapid initial elimination of most of the arsenic administered, the elimination of the remaining portion of the dose is a very slow process. The elimination rate constant calculated for this second phase of As elimination ($0.023\,h^{-1}$), corresponds to a half-life of 30 h.

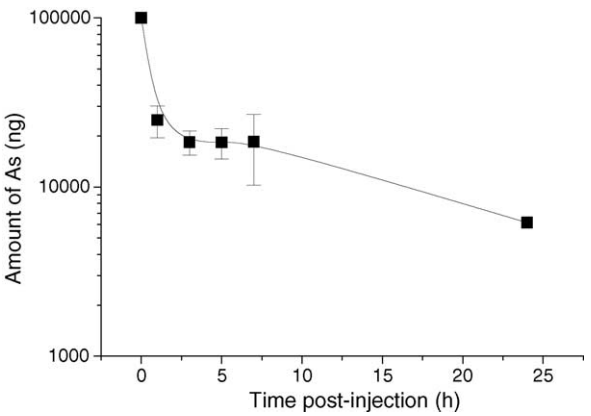

Fig. 2. Time-course of clearance of arsenic from the body of balb-c mice after intraperitoneal injection of arsonoliposomes (C16/PC/Chol 8:12:10 mol/mol/mol) at a dose equivalent to 5 mg As/kg of body weight. Each point is the cumulative amount of As (ng) remaining in the body of mice (mean value plus standard deviation calculated from – at least – four different animals).

From the distribution results and the corresponding tissue mean weight, we calculated the concentration of arsenic in each tissue (% dose/g tissue), which is presented in Table 3. As observed from these results, the tissues with the highest arsenic concentration during the study period are: spleen–kidney–stomach, followed by lung, liver, intestines–heart, carcass + skin and finally blood. Skin was not separated from carcass, so we do not have a picture for each separate portion of this sample. In addition, the weight of the combined sample was very large, decreasing the final concentration of arsenic that was too low for quantification in the 24-h sample.

From the values presented in Table 3, we observe the different clearance patterns of arsenic from each organ. The elimination rate constant of arsenic from various tissues was calculated from the three or four last data points of the log-concentration–time curves. Linear correlations could be derived only in the cases of liver, kidney and lung (R ranging between –0.9611 [for kidney] and –0.998 [for liver]). The slope values obtained (elimination rate constant, K_{el}), reveal that clearance or arsenic from liver ($K_{el\text{-}liver} = 0.115\,h^{-1}$) is faster compared to kidneys ($K_{el\text{-}kidney} = 0.051\,h^{-1}$) and lungs. Especially the lungs clear arsenic very slowly, $K_{el\text{-}lung} = 0.037\,h^{-1}$ which, corresponds to a half-life-in-lung of approximately 19 h.

4. Discussion

Arsonoliposomes have been previously shown to possess interesting cancerostatic (Gortzi et al., 2002, 2003) and very recently antiprotozoal activity

Table 3
Concentration of As in tissues and carcass/skin of mice at various time points following i.p. administration of arsonoliposomes (containing 5 mg/kg arsenate)

Time (h)	Blood	Liver	Kidney	Lung	Spleen	Heart	Stomach	Intestines	Carcass and skin
1	0.146 (0.016)	2.97 (0.32)	5.38 (0.80)	1.80 (0.15)	5.26 (0.73)	1.13 (0.19)	6.09 (0.92)	1.39 (0.23)	0.93 (0.16)
3	0.269 (0.058)	2.40 (0.57)	9.79 (1.51)	2.00 (0.47)	5.86 (0.98)	1.15 (0.30)	2.86 (0.47)	0.191 (0.028)	0.75 (0.11)
5	0.298 (0.043)	2.50 (0.76)	5.22 (0.89)	2.62 (0.53)	5.81 (0.69)	1.56 (0.28)	3.07 (0.62)	0.378 (0.022)	0.74 (0.14)
7	0.298 (0.044)	1.83 (0.32)	4.77 (0.28)	2.64 (0.24)	4.48 (0.72)	2.47 (0.42)	2.49 (0.59)	2.00 (0.24)	0.80 (0.17)
12	0.381 (0.10)	0.981 (0.036)	2.87 (0.36)	2.12 (0.29)	6.82 (1.13)	1.186 (0.077)	3.40 (0.68)	2.06 (0.42)	(0.84) (0.14)
24	0.322 (0.073)	0.273 (0.041)	2.00 (0.24)	1.35 (0.30)	2.68 (0.42)	1.12 (0.17)	1.66 (0.10)	1.676 (0.061)	BDL

BDL: below detection limit. Results are expressed as mean% of dose/g tissue (±S.D.). Each point is the mean of four values.

(Antimisiaris et al., 2003) in in vitro studies. In the later study the in vivo distribution of arsenic after i.p. injection of arsonoliposomes in balb-c mice was evaluated, however due to the lack of an appropriate analytical technique to measure arsenic in our laboratory, the results of the preliminary study could only provide proof that part of the liposomes were accumulating in the liver. Herein, after setting up a very sensitive atomic absorption spectrophotometric technique, suitable to measure arsenic in tissues after acidic digestion, we evaluated the distribution of arsenic in respect to time post-injection in balb-c mice. The distribution results reveal that arsenic is accumulating in high amounts in various tissues as, kidney, spleen, lung, liver, stomach, heart, carcass + skin and intestines.

In general the distribution of arsenic in tissues is a complex mechanism not fully elucidated. Indeed, it should be considered as a combination of the metabolism of the arsenic-containing compound and the relative kinetics of each metabolite. In addition, the metabolism of aronolipids may be completely different from that of iAs, as evaluated recently for arsenosugars (Devalla and Feldmann, 2003). Thereby, any attempt to correlate the results presented here with studies performed with inorganic arsenic or arsenate compounds, could only provide possible answers to the question: *Are the kinetics of arsenic modulated as a results of administration in the form of a lipid* (*arsonolipid*) *incorporated in vesicles*? In a study performed recently in order to evaluate possible strain dependency on the disposition of inorganic arsenic in mice (Hughes et al., 1999) it was found that the clearance of [^{73}As]arsenate after i.p. administration in three different strains of mice was strain independent. In the same study the researchers measured the distribution of As in tissues of mice 24 h after i.p. administration of 5 mg As/kg of [^{73}As]arsenate (the same dose we used). If we compare the results of this later study with our results, it is easily observed that in all cases of tissues the concentration of As is substantially higher after arsonoliposome administration. Indeed, after administration of iAs, the concentrations of As in liver, kidney and lung 24-h post-injection range between 0.12–0.14, 0.17–0.19 and 0.08–0.1 [mean% of dose/g tissue], respectively for each tissue. These values are more than 2× lower for liver and more than 10× lower for kidneys and lung compared with the results obtained here, after administration of arsonoliposomes. This may serve as an indication of increased retention of arsenic in tissues, which may be related to the fact that in this study arsenic was administered in the form of a lipid incorporated in vesicles.

Indeed the rapid accumulation of As in liver and spleen (in which a high concentration remains even 24-h post-administration) correlates well with biodistribution profiles demonstrated when conventional (non-pegylated) small sized liposomes, are administered by i.p. injection. Intraperitoneal administration of liposomal formulations of anti-cancer drugs is generally preferred, since accumulation of the drug in tumours is higher, compared to that obtained after i.v. administration (Sadzuka et al., 2000), and at the same time, plasma drug concentration is many times lower, minimizing toxicity (Sadzuka et al., 1997; Marchettini et al., 2002). In a previous study, in which the tissue distribution of adriamycin was evaluated after i.v. and i.p. injection of conventional and pegylated drug-encapsulating liposomes (Sadzuka et al., 1997), the amount of drug in plasma was substantially lower after i.p. injection (compared to iv), increased between 2- and 4-h post-injection and remained more or less constant up to 24 h. This pattern is very similar to the blood distribution

pattern observed here for arsenic (Table 3). In addition, the accumulation of drugs in the liver after i.p. administration of conventional liposomal formulations, is initially (in the first few hours) substantially lower compared to i.v. administration, but remains almost constant between 2- and 6-h post-injection, in good correlation again, with what we observed here for arsenic (Table 3) Thereby, our results may indicate that the distribution of arsenic after administration of arsonoliposomes by i.p. injection, is mainly governed by the vesicle distribution and that any effect of arsonoliposome disruption and subsequent metabolism of arsonolipids is minimal.

The values measured in stomach and intestines, when compared between them, may indicate that a portion of the vesicles is digested, but not absorbed (a sharp initial decrease of arsenic concentration in stomach and intestines is followed by an increase in the intestines after 7-h post-injection, while the stomach concentration remains more or less constant between 3- and 12-h post-injection).

The slow clearance of arsenic from lung demonstrated here (that translates in a lung-half-life of approximately 19 h) is substantially slower than the previously reported lung-clearance (half-life of arsenic of 13 h) calculated after intratracheal installation of industrial dust (particles) in hamsters (Leffler et al., 1984). While it has been reported previously (Rhoads and Sanders, 1985) that after inhalation or intratracheal instillation in rats, a small fraction of arsenic remains in the lungs for a long period (400 days), we should not exclude the possibility that arsonoliposomes may remain in the lungs for prolonged time periods due to an increase in their size after aggregation. Indeed, very recently (Fatouros et al., 2005) we demonstrated that arsonoliposomes aggregate and subsequently fuse to larger particles in presence of divalent cations as Ca^{2+}. If this is taking place in the lungs, it may be a possible explanation of the increased lung-half-life of As measured here. However, this is just a suggestion and we do not have any further proof to support it.

Concluding, we evaluated here the tissue distribution of arsenic after i.p. administration of arsonoliposomes in mice. Although the distribution profiles are in line with previously reported values for liposomal drugs administered by the same route, we cannot be absolutely sure that the time course of the distribution of arsenic measured herein is not a combination of vesicle distribution and arsonolipid metabolism. Nevertheless, these results are the first in vivo results obtained with arsonoliposomes and will serve as a basis for comparison of future in vivo studies with other types of arsonoliposomes. In addition, it is important to state that no acute toxicity was observed and the body weight and organ weight of the mice receiving this rather high dose of arsenate was not altered. Nevertheless, it is essential that a histological evaluation of toxicity is carried out, especially in the organs in which high percent of arsenic seems to be present for extended time periods, as lungs, spleen, kidneys and liver.

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